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Prevalence Studies of GB Virus-C Infection Using Reverse Transcriptase-Polymerase Chain Reaction

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Among the three recently described GB viruses (GBV-A, GBV-B, and GBV-C), only GBV-C has been linked to cryptogenic hepatitis in man. Because of the limited utility of currently available research tests to determine antibody response to GBV-C proteins, the prevalence of GBV-C RNA in human sera was studied using reverse transcription-polymerase chain reaction (RT-PCR). The prevalence of GBV-C is higher among volunteer blood donors with elevated serum alanine aminotransferase (ALT) levels (3.9%) than among volunteer blood donors with normal ALT levels (0.8%). Higher rates were also noted among commercial blood donors (12.9%) and intravenous drug users (16.0%). GBV-C was frequently detected in residents of West Africa, where the prevalence was >10% in most age groups. Approximately 20% of patients diagnosed with either acute or chronic hepatitis C virus (HCV) were found to be positive for GBV-C RNA. In addition, GBV-C RNA sequences were detected in individuals diagnosed with non-A-E hepatitis, with clinical courses ranging from mild disease to fulminant hepatitis. Fourteen of sixteen subjects with or without clinically apparent hepatitis were positive for GBV-C RNA more than 1 year after the initial positive result.

KEY WORDS: hepatitis, GBV-C, HCV

INTRODUCTION

Three new flavi-like viruses (GBV-A, GBV-B, and GBV-C), have recently been identified, each having a single strand positive-sense RNA genome of approximately 10 kb [Simons et al., 1995a,b; Muerhoff et al., 1995; Leary et al., 1996a]. GBV-A and GBV-B were isolated from serially passaged tamarin serum containing the "GB agent" [Simons et al., 1995a], which originated from the serum of a surgeon (initials G.B.) diagnosed with acute hepatitis [Deinhardt et al., 1967]. Subsequently, GBV-A-like sequences were detected in tamarin sera prior to experimental exposure to the GB agent, suggesting that GBV-A or its variants is a virus(es) of nonhuman primates [Schlauder et al., 1995b]. Although GBV-B clearly induces hepatitis in tamarins [Schlauder et al., 1995a], and presumably had its origins in human serum, GBV-B sequences have not yet been detected in human sera [Simons et al., 1995b]. GBV-C sequences were first discovered in human serum by performing reverse transcription-polymerase chain reaction (RT-PCR) with degenerate oligonucleotides derived from the highly conserved helicase domains of GBV-A, GBV-B, and HCV [Simons et al., 1995b]. Additional studies confirmed that GBV-C is frequently detected in the serum of intravenous drug users (IVDUs) and in some patients with cryptogenic hepatitis [Simons et al., 1995b; Yoshiba et al., 1995; Leary et al., 1996b].

Recently, a virus similar to GBV-C, designated hepatitis G virus (HGV), was identified, and it also appears to be prevalent both among individuals with risk factors for exposure to parenterally transmitted viruses and among individuals with cryptogenic hepatitis [Linnen et al., 1996]. Sequence analyses indicate that HGV and GBV-C are highly related (85% nucleotide identity, 95% amino acid identity) and represent members of the same virus group [Zuckerman, 1996]. Based on sequence analysis

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of the 5′ end of the genome, the GBV-C viruses have been provisionally subdivided into five genotypes [Muerhoff et al., 1996a]. The prototype GBV-C virus has provisionally been classified as genotype 1b, and the HGV prototype virus has been classified as genotype 2a.

Because of the limited utility of immunoassays for detecting antibodies among GBV-C RNA-positive individuals, the present study utilized RT-PCR to study the prevalence of GBV-C in human populations. Various groups were selected for this study ranging from volunteer blood donors, assumed to be "low risk" for parenteral exposure to potential hepatotropic viruses, groups such as IVDUs, individuals diagnosed with cryptogenic hepatitis, and others believed to be "at risk" for exposure to GBV-C. The prevalence rates for GBV-C RNA ranged from <1% in volunteer blood donors to 15–20% in IVDUs and HCV-seropositive individuals. Furthermore, in this paper we report that GBV-C establishes a persistent viremia, which may be detectable for 1 year or more following infection.

MATERIALS AND METHODS

RT-PCR

Nucleic acid was prepared from 25–70 μl of serum or plasma that had been stored at −70°C. The nucleic acid was extracted using the Ultraspec RNA Isolation System (Biotecx), RNA/DNA Extraction Kit (United States Biochemical; USB), or QIAmp HCV Kit (QIAGEN) following the manufacturer's recommended protocol. The protocol for preparing cDNAs PCR and the analysis of PCR products was performed as previously described [Leary et al., 1996b; Muerhoff et al., 1996b]. Briefly, precipitated total nucleic acid was resuspended in 3.75 μl water, or 3.75 μl of the column eluate from the QIAGEN column, and was reverse transcribed with random primers in a 25 μl reaction (RNA-PCR Kit; Perkin-Elmer). One-fifth of the cDNA reaction was amplified in each 25 μl PCR. One reaction uses two nonoverlapping sets of primers, one from the NS3 region [Leary et al., 1996b], and the other reaction uses primers from the 5′-untranslated region [Muerhoff et al., 1996b]. PCR products were separated on a 2% NuSieve 3:1 (FMC) agarose gel followed by Southern blot analysis with radiolabeled probes specific for the appropriate region. A serum or plasma sample was considered GBV-C RNA positive if both primer sets resulted in detection of a hybridizable product of the expected size.

Recombinant Proteins

GBV-C recombinant proteins C6, C10, and C12 were expressed as CKS fusion proteins in *Escherichia coli*, grown and harvested as previously described [Pilot-Matias et al., 1996]. Two additional regions of GBV-C were utilized in this study. Recombinant protein C29 was produced as a CKS fusion protein in *E. coli* as described above, and recombinant protein C30 was produced as a nonfusion protein in *E. coli* using the lambda pL promoter as described elsewhere [Devare et al., 1984]. The regions of the GBV-C polyprotein represented by each of the recombinant proteins are presented in Table I.

Protein Purification

Bacterial cells were lysed at pH10 in the presence of various protease inhibitors. The cell lysates were then centrifuged, and the resulting pellet was subjected to a series of detergent and buffer washes to remove nonspecific proteins. After solubilization in buffer containing sodium dodecyl sulfate (SDS), the CKS protein typically represented between 40% and 60% of the total protein as evaluated by SDS-polyacrylamide gel electrophoresis (PAGE) and gel densitometry. The solubilized proteins were further purified by gel filtration chromatography (Sephacryl S-300HR; Pharmacia, Uppsala, Sweden). Fractions were analyzed via SDS-PAGE, pooled, and evaluated for purity using gel densitometry. In general, the proteins were evaluated as being at least 80% homogeneous. Final purified proteins were used to coat polystyrene beads for evaluation in enzyme-linked immunosorbent assays (ELISAs).

ELISAs

Conditions were optimized for coating 0.25-inch polystyrene beads with various concentrations for each of the purified proteins. The format for the ELISAs entails contacting the antigen-coated solid phase with serum or plasma prediluted in specimen diluent (buffered solution containing animal sera and nonionic detergents) and detecting bound immunoglobulins with a second antibody (horseradish peroxidase-labeled goat antihuman immunoglobulin) as previously described [Dawson et al., 1992].

For each of the five separate ELISAs, a preliminary cutoff value was set to separate those specimens that presumably contain antibodies to the GBV recombinant protein from those that appeared to be unreactive. Specimens that were initially reactive were retested in duplicate. If one or more of the duplicate wells was reactive, the specimen was considered positive.

Specimens

Blood donors. Among the sera tested are those from donors assumed at "low risk" for exposure to GB viruses. These specimens were obtained from volunteer blood donors from the United States and Sweden with normal serum alanine aminotransferase (ALT) levels (less than or equal to 45IU/L) and were unreactive for hepatitis B surface antigen (HBsAg), antibodies to hepatitis B core (anti-HBc), and antibodies to hepatitis C virus (HCV; second-generation HCV EIA) by commercial tests obtained from Abbott Laboratories (North Chicago, IL). Those sera considered to be "at risk" for exposure to GB viruses included specimens obtained from volunteer blood donors with elevated ALT values and from commercial plasma donors.

IVDUs. Sera from 100 patients randomly selected from a pool of 438 IVDUs were retrospectively screened for GBV-C RNA. These individuals were considered at "high risk" for exposure to parenterally transmitted vi-

TABLE I. GBV-C Recombinant Proteins Utilized in ELISAs

GBV-C recombinant protein	Region of GBV-C polyprotein	GBV-C amino acid residues encoded[a]
C6	NS2/3	875–1302
C10	NS5A	1969–2151
C12	NS5A/B	2330–2585
C29	E2	272–483
C30	NS5A	1970–2336

[a]Pilot-Matias et al., 1996.

ruses and were selected for this study because of their high rates of exposure to HBV as determined by anti-HBc (74.7%) and HCV (99.0%) [Lentino et al., 1991]. Among the 96 specimens for which complete data was available, 95 were antibody positive to HCV. Statistical evaluations for dual infections of GBV-C and HBV or HCV viruses were performed using χ^2 and exact probability testing as described elsewhere [Swinscow, 1982].

Residents of West Africa. A collection of specimens was available as part of an ongoing hepatitis virus prevalence study [Martinson et al., 1996]. A subgroup of these specimens was tested for GBV-C RNA by RT-PCR.

Hepatitis specimens.

Acute/chronic HCV. A number of patients previously diagnosed with acute or chronic HCV infection were tested for GBV-C RNA. Twenty individuals from site 1 (provided by Dr. Dwain Thiele, University of Texas, Southwestern Medical Center, Dallas) were diagnosed as having acute HCV. Eight specimens obtained from site 2 (provided by Dr. Jon Rosenblatt, Mayo Clinic, Rochester, MN) were diagnosed with chronic HCV infection.

Acute non-A-E hepatitis (site 1). Serum was available for patients who had symptomatic acute illnesses, consistent with a diagnosis of acute viral hepatitis. The patients had no evidence suggestive of chronic liver disease and had serum aminotransferase elevations greater than ten times the upper limit of normal and were nonreactive for HBsAg and nonreactive for antibodies HCV and for IgM-class antibodies to hepatitis A virus (HAV) and HBc. Patients were excluded if aminotransferase abnormalities resolved in <2 weeks after the first observation. Patients with a history consistent with drug- or toxin-induced liver disease or congestive heart failure or evidence of choledocholithiasis, other disseminated viral or bacterial infections, or other diseases that can result in elevated aminotransferase levels were also eliminated from the study. Those with severe acute non-A-E hepatitis were characterized as having serum bilirubin values >15 mg/dl or prothrombin times prolonged by more than 3 seconds.

Non-A-E hepatitis (site 2). Serum was available from 149 patients who had a variety of acute and chronic hepatic disorders for whom viral hepatitis was initially considered in the differential diagnosis. Clinical specimens were unreactive for HBsAg, hepatitis B "e" antigen (HBeAg), antibodies to HBc or HCV, and IgM-class antibodies to HAV. Specific diseases include the following disorders in which etiology was not identified: abnormally elevated serum liver enzyme values [aspartate aminotransferase (AST) and ALT], past history of hepatitis, past or present history of jaundice, acute non-A, non-B, non-C (NANBNC) hepatitis, and fulminant hepatitis. Chronic hepatic disorders include primary sclerosing cholangitis, cryptogenic cirrhosis, chronic liver disease of unknown etiology, chronic active hepatitis/autoimmune hepatitis, and hepatobiliary cancers. Also included was a group of patients who presented with a variety of constitutional symptoms for whom viral hepatitis was considered in the differential diagnosis.

RESULTS

Serologic Studies

Recombinant proteins from different regions of the GBV-C genome were expressed in *E. coli* (see Table IV). Each of the recombinant proteins was purified and utilized in an ELISA to determine antibody response among individuals who were positive for GBV-C RNA by RT-PCR. Among commercial blood donors from the United States, 1 of 13 (7.7%) GBV-C RNA-positive specimens was positive for antibodies to one or more GBV-C proteins. In comparison, 7 of 18 (38.9%) West African samples and 3 of 12 (25%) individuals with non-A-E hepatitis were positive for antibodies to GBV-C proteins (Table II). In total, 11 of the 43 (25.6%) specimens that were PCR positive for GBV-C RNA were also antibody positive for GBV-C proteins. There was no single consistent antigen recognized, and, in all cases but one, individuals produced antibodies that were reactive against only one of the GBV-C recombinant proteins. Therefore, prevalence studies were conducted using RT-PCR to detect the presence of GBV-C RNA.

GBV-C RNA Studies

Blood donors. Among volunteer blood donors with normal ALT values, 1 of 127 (0.79%) was GBV-C RNA positive. In contrast, 8 of 204 (3.9%) volunteer blood donors with elevated ALT values were positive (Table III), and 12 of 93 (12.9%) commercial blood donors were positive. The prevalence of GBV-C in commercial blood donors is significantly higher than that observed in volunteer blood donors with normal ALT values ($p < 0.001$) and with elevated ALT values. There was no significant differences between the volunteer donors with normal ALT values compared to those with elevated ALT values ($p < 0.5$, >0.1).

West Africa. Forty-two of two hundred ninety (14.2%) of the specimens from West African sera were

TABLE II. Detection of Antibodies to GBV-C Recombinant Proteins Among Individuals Viremic for GBV-C*

Category	No. antibody positive/total (%)	GBV-C recombinant proteins recognized by each seropositive subject
Plasmapheresis donors	1/13 (7.7%)	C29, C30
Non-A-E hepatitis	3/12 (25.0%)	C29 C10 C10
Residents of West Africa	7/18 (38.9%)	C29 C10 C30 C10 C6 C6 C30
Total	11/35 (31.4%)	

*ELISAs were developed for several different recombinant proteins—C3, C6, C10, C29, and C30—as described elsewhere [Pilot-Matias et al., 1996].

TABLE III. Prevalence of GBV-C Viremia Among a Variety of Blood Donors*

Category	Positive/total (%)
Volunteer blood donors with normal ALT values	1/127 (0.79%)
Volunteer blood donors with elevated ALT values	8/204 (3.92%)
Commercial blood donors	12/93 (12.90%)

*All volunteer blood donors were screened negative for HBsAg and also were negative for antibodies to HCV, HIV, and HBc. Commercial blood donors were screened negative for HBsAg and for antibodies to HCV and HIV.

TABLE IV. Seroprevalence and 95% Confidence Intervals for GBV-C RNA Among West African Residents

Age groups (years)	GBV-C RNA positive/total (%)		
	Males	Females	Total
1–5	1/13 (7.7)	1/12 (8.3)	2/25 (8.0)
6–10	8/50 (16.0)	5/57 (8.8)	13/107 (12.6)
11–15	7/51 (13.7)	8/50 (16.0)	15/101 (14.9)
16–20	3/11 (27.3)	2/11 (18.2)	5/22 (22.7)
21–40	1/6 (16.7)	3/16 (18.8)	4/22 (18.2)
>40	0/5 (0)	3/8 (37.5)	3/13 (23.1)
Total	20/135 (14.8)	22/135 (15.8)	42/290 (15.2)
Confidence intervals	8.8–20.8%	10.1–21.5%	10.8–10.6%

PCR positive for GBV-C RNA (Table IV). Although there was no difference in prevalence between the two genders, there was an increase in prevalence with advancing age ($P = 0.04$). The seroprevalence of antibodies to HCV was 11.1% for GBV-C RNA-positive individuals and 6.1% for GBV-C RNA-negative individuals. However, the number of persons with antibodies to HCV was too small for the difference in seroprevalence to reach significance ($P = 0.30$). The seroprevalence of exposure to hepatitis B virus was 60.1% among the GBV-C RNA-positive individuals and 70.3% among GBV-C RNA-negative individuals, and this difference was not significant ($P = 0.24$).

Specimens from HCV-seropositive subjects. Six of twenty-seven (22.2%) patients with acute HCV were positive for GBV-C RNA and 2 of 8 (25%) patients with chronic HCV were positive for GBV-C RNA (Table V). Among a population of 95 IVDUs who were seropositive for HCV, 15 (15.8%) were positive for GBV-C RNA. A total of 64 of the 95 (67.3%) specimens were positive for anti-HBc. There was a significantly lower incidence ($P = 0.05$) of anti-HBc reactivity in individuals with GBV-C RNA than among those without evidence of GBV-C infection.

Non-A-E hepatitis/liver disease. From one site, a total of 37 cases of acute non-A-E hepatitis were classified for the severity of disease. For the cases of mild non-A-E hepatitis, 1 of 16 (6.7%) was positive for GBV-C RNA (Table VI). Among cases of severe disease, not associated with progression to subfulminant or fulminant hepatitis, none of 14 patients were positive for GBV-C. Among seven cases of subfulminant or fulminant hepatitis, 3 of 7 (42.9%) were positive for GBV-C.

TABLE V. Prevalence of GBV-C in Individuals Seropositive for Hepatitis C Virus

Category	GBV-C PCR results positive/total (%)
Acute HCV	6/27 (22.2%)
Chronic HCV	2/8 (25.0)
Intravenous drug users	15/95 (15.82)

TABLE VI. Prevalence of GBV-C in Non-A-E Hepatitis

Category	GBV-C PCR results positive/total (%)
Site 1: Acute non-A-E hepatitis	
Mild	1/16 (6.7)
Severe (excluding fulminant and subfulminant cases)	0/14 (0)
Fulminant/subfulminant cases	3/7 (42.9)
Total	4/37 (10.8)
Site 2: Non-A-E liver disease	
Elevated liver function test values	3/67 (4.5)
Crytogenic cirrhosis	5/34 (14.7)
History of hepatitis	1/7 (14.3)
Chronic active hepatitis	1/5 (20.0)
Chronic liver disease	1/10 (10.0)
Sclerosing cholangitis/biliary cirrhosis	0/11 (0)
Acute hepatitis/fulminant hepatitis	2/9 (22.2)
Hepatocellular carcinoma	0/2 (0)
Jaundice of unknown etiology	0/4 (0)
Total	13/149 (8.7)

At a second site, 13 of 149 (8.7%) cases of non-A-E liver disease were positive for GBV-C RNA (Table VI). These specimens were obtained from individuals with different diagnoses of liver disease. Five of thirty-four (14.7%) patients with cryptogenic cirrhosis were GBV-C RNA positive. Two of nine (22.3%) patients with acute and/or fulminant hepatitis were GBV-C RNA positive.

Evidence for Persistent Viremia

Serial bleeds were available for five GBV-C RNA-positive IVDUs. All five individuals remained positive for the entire follow-up period, 24 months for one subject, 33 months for a second subject, and 36 months for the remaining subjects (Table VII). Results of representative serial bleeds from one of the IVDU subects whose sera were tested for GBV-C RNA every 3 months over a 33 month period are shown in Figure 1.

Follow-up specimens were obtained between 16 and 20 months after the initial bleed date from nine individuals from West Africa. Seven of the nine individuals were again PCR positive nearly 1.5 years after the initial test. In addition, two individuals who were GBV-C RNA positive and diagnosed with non-A-E hepatitis were tested on two bleed dates 1 year apart. For both patients, GBV-C RNA was detected for at least 1 year after the first positive result.

DISCUSSION

A common feature among the three newly discovered GBV group of viruses has been the difficulty in detecting a consistent immune response in infected subjects. As was previously reported, tamarins infected with GBV-A did not produce detectable antibodies to any of the gene products utilized as antigenic targets, whereas tamarins infected with GBV-B produced a transient immune response which became undetectable within 2 months after clearance of the virus [Schlauder et al., 1995a; Pilot-Matias et al., 1996]. Similarly, the lack of a consistent detectable antibody response in GBV-C-infected individuals was noted both in a previous study by Pilot-Matias et al. [1996] and in the present study. Thus, RT-PCR was utilized to determine the prevalence of GBV-C in different populations. The current antigenic targets consist of nonstructural proteins or structural proteins (such as the putative E2 protein), which were expressed in *E. coli* cells as fusion proteins. The expressed proteins accumulate as inclusion bodies in the bacterial cells and require denaturing conditions for purification. Furthermore, the recombinant proteins utilized were not designed with the proposed amino or carboxyl termini of fully processed viral proteins, nor were the proteins glycosylated. This being the case, these recombinant proteins are less likely to represent their native conformation. The correlation between antibody to GBV-C proteins and GBV-C RNA detection may be improved once antigenic targets that more closely simulate the viral proteins encountered in vivo become available.

The inherent difficulty in employing RT-PCR to study prevalence is that individuals who have recovered from GBV-C infection and are no longer viremic will not be included in the prevalence data. Likewise, GBV-C viremic individuals who test as PCR negative because of either low viral load or the quality of specimens (specimen storage conditions, etc.) will not be counted in the current prevalence study. Thus, utilizing RT-PCR to monitor GBV-C exposure will likely underestimate the true prevalence of GBV-C.

The high prevalence of GBV-C noted among IVDUs supports previous data indicating that GBV-C/HGV can be parenterally transmitted to recipients of blood or blood products [Linnen et al., 1996]. Recent data indicate that GBV-C/HGV can also be vertically transmitted [Feucht et al., 1996]. In the current study, nearly 10–15% of children from West Africa under 10 years of age were GBV-C RNA positive. It is unlikely, however, that vertical transmission is the sole route of transmission in this population, because the prevalence rates increase with age. It is possible that GBV-C can also be spread horizontally or can be transmitted by mosquitoes in regions of the world where mosquitoe-borne viral infections are common [Holland, 1996; Zanetto et al., 1996]. Additional studies are needed to determine the routes by which GBV-C can be transmitted.

In contrast to data from a previous study [Linnen et al., 1996], data presented in Table III indicate that volunteer blood donors with elevated ALT values have a higher prevalence of GBV-C than blood donors with normal ALT levels. This supports the idea that GBV-C plays a role in liver disease. The prevalence rate among commercial donors is also higher than that noted among volunteer donors, supporting the earlier observation

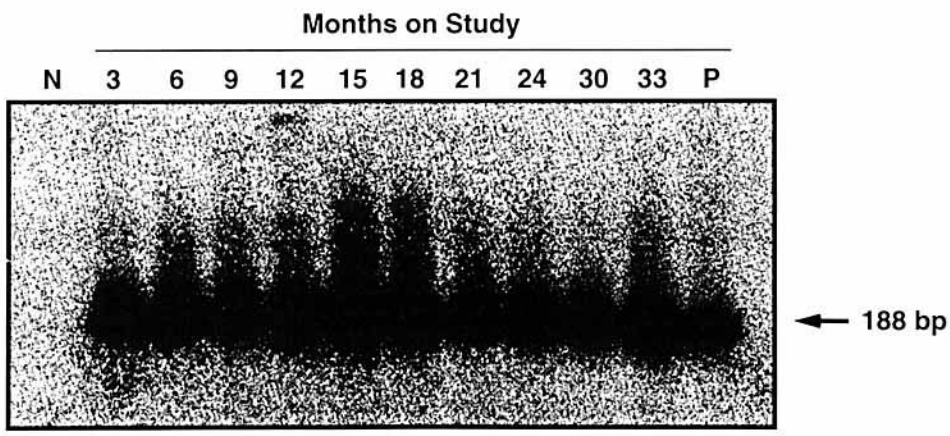

Fig. 1. Southern blot hybridization of serial bleeds from an IVDU. Sera were obtained every 3 months over a 33 month period from an IVDU, initially found positive for GBV-C, utilizing primers from the NS3 region of the GBV-C genome.

TABLE VII. Persistent Viremia in GBV-C RNA-Positive Subjects

Category	No. of subjects GBV-C RNA positive/total tested	
	12–18 Months after first PCR-positive result	24–36 Months after first PCR-positive result
Intravenous drug users	5/5	5/5
West Africans	7/9	Not done
Non-A-E hepatitis	2/3	Not done

that plasma pools are frequently positive for GBV-C sequences [Nubling and Lower, 1996]. These data suggest that blood and/or blood products generated from volunteer donors and paid donors represent a risk to recipients for transmission of GBV-C.

Subsequent to the discovery of HCV and HEV [Choo et al., 1989; Reyes et al., 1990] and the development of tests to determine exposure to these viruses, there have been several reports of community-acquired hepatitis [Alter et al., 1992; Tassopoulous et al., 1994] and transfusion-associated hepatitis [Thiers et al., 1993; Peters et al., 1993], which implicate a putative unknown viral etiologic agent. Recent data indicate that GBV-C may be responsible for some cases of posttransfusion hepatitis [Linnen et al., 1996] and for some cases of fulminant hepatitis [Mishiro et al., 1995]. In the current study, the detection of GBV-C has been linked to fulminant hepatitis and chronic non-A-E hepatitis. Frequently, viruses that establish persistent infection produce chronic diseases in their hosts. It has been observed that GBV-C frequently establishes a long-term association with infected hosts, with most of the individuals remaining viremic for several years. Taken together, the prevalence data for GBV-C suggest that, as with HCV, GBV-C can be detected in individuals with a wide spectrum of health profiles, ranging from individuals with normal ALT levels (e.g., volunteer blood donors) to individuals with fulminant hepatitis. The role of continuing viral presence in inducing chronic or progressive diseases in infected hosts is not clear at present.

As was noted in a previous study, GBV-C RNA is frequently detected among individuals who are seropositive for HCV [Linnen et al., 1996]. We also noted that approximately 20% of the HCV cases tested in the current study were GBV-C RNA positive. This might indicate similar routes of infection. The potential role of GBV-C in influencing the pathogenicity of HCV is not clear at present.

Less than 10% of the cases classified as having non-A-E hepatitis were positive for GBV-C RNA. It is possible that the prevalence of GBV-C is underestimated as a result of the failure to eliminate autoimmune hepatitis, alcoholic hepatitis, as well as hepatitis due to drugs and a variety of toxins from non-A-E hepatitis cases. An alternate explanation is that there is at least one as yet undiscovered viral agent responsible for non-A-E hepatitis. These cases may be caused by agents such as those proposed for giant cell hepatitis [Phillips et al., 1991] and the agent proposed for fulminant hepatitis [Fagan et al., 1992].

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